

Universidade de Lisboa

Faculdade de Ciências da Universidade de Lisboa

Departamento de Biologia Vegetal



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metabolites in a model of neurodegeneration based on mice
neurons primary culture**

Inês de Sousa Costa

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2014

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“Success is not final, failure is not fatal: it is the courage to continue that counts.”

Winston Churchill (1874-1965)

ABSTRACT

Neurodegenerative diseases represent a large and heterogeneous group of neurological disorders with increasing incidence associated with aging. Oxidative stress and glutamate excitotoxicity are thought to be one of the main contributing factors to neurodegeneration. The ingestion of fruits and vegetables have been associated to a decreased risk of neurodegenerative and cardiac diseases amongst others, which justifies the increased interest for the preventive properties of (poly)phenols from berry fruits. Thus, the aim of this work was to validate the neuroprotective effect of blackberries digested metabolites in a model of neurodegeneration based on mice neurons primary culture.

Establishment of cerebellar granule cells in culture was performed from 7 day-old mice. Initially, an optimization of some media components was performed and integrity and functionality of primary neurons was confirmed. To mimic neurodegeneration, glutamate was chosen as the chemical stimulus under 90 μM , as it has given the ideal percentage of death to allow cell response to digested extracts. In order to evaluate the neuroprotective effect of blackberries digested metabolites, different concentrations, close to the physiological range concentrations of phenolic compounds detected in human plasma, were tested, and as no toxic effects were detected, concentrations from 0.25-1 $\mu\text{g GAE mL}^{-1}$ were chosen for further cytoprotective assays. Significantly similar neuroprotective effects were obtained for the three concentrations tested when compared to cells exposed only to glutamate. Mitochondrial electron transport chain complexes were analyzed at protein level through western blot. Preliminary analysis at DNA level for further quantitative PCR was also initiated.

In conclusion, this work allowed the establishment of primary cultures of cerebellar granule cells and the validation of blackberries digested metabolites neuroprotective effect. It was attempted mitochondrial complexes I, II and IV as the most affected under glutamate induced excitotoxicity, with a partial recovery of complex IV incubations with digested extract.

Keywords: neurodegenerative disorders, cerebellar granule cells, blackberries digested metabolites, (poly)phenols, mitochondria

RESUMO

As doenças neurodegenerativas, como são exemplo as doenças de Alzheimer, Parkinson, Huntington e Esclerose Lateral Amiotrófica, representam um amplo e diversificado grupo de distúrbios neurológicos com características clínicas e patológicas bastante heterogêneas. Esta diversidade é resultado do envolvimento de subconjuntos específicos de neurónios presentes em sistemas funcionais também eles específicos. O stress oxidativo tem sido descrito como um importante fator que condiciona o envelhecimento e as alterações cognitivas associadas a este tipo de doenças, podendo ser provocado por várias razões, incluindo a acumulação de glutamato extracelular.

As células neuronais são altamente vulneráveis ao stress oxidativo não só devido às suas necessidades energéticas elevadas e ao elevado consumo de oxigénio, mas também por outro lado por apresentarem baixos níveis de antioxidantes endógenos, o que justifica a necessidade de sistemas biológicos de defesa (antioxidante) capazes de manter o balanço fisiológico relativamente à formação/eliminação das espécies reativas de oxigénio/azoto (ROS/RNS). O glutamato é o neurotransmissor endógeno mais predominante no cérebro humano, sendo as suas respostas mediadas por dois tipos de recetores pós-sinápticos: os ionotrópicos (*N*-metil-*D*-aspartato (NMDA), ácido α -amino-3-hidroxi-5-metil-4-isoxazolepropiónico (AMPA), cainato) e os metabotrópicos. A acumulação excessiva de glutamato extracelular na fenda sináptica por um período prolongado de tempo, que tem por consequência danos neuronais e morte celular, refere-se ao processo designado por excitotoxicidade.

A mitocôndria representa um dos organelos mais críticos para a sobrevivência da célula, pois apresenta o maior consumo de oxigénio celular, contém DNA próprio e sofre alterações contínuas através dos processos de fissão e fusão consoante as necessidades/condições energéticas da célula. A fosforilação oxidativa, processo através do qual a célula consegue produzir energia, ocorre através de cinco complexos multiproteicos e dois transportadores de eletrões que constituem a cadeia transportadora de eletrões. Esta constitui a maior fonte de ROS, que a par das anomalias mitocondriais representam fatores de grande importância no processo de envelhecimento e patogénese das doenças neurodegenerativas.

A manutenção das faculdades cognitivas, que por sua vez é sinónimo de qualidade de vida, é influenciada por inúmeros fatores, sendo a nutrição um dos principais. Nesse sentido, os (poli)fenóis são moléculas que têm vindo a ser bastante investigadas nesta área. Estruturalmente estes contêm um ou mais anéis de benzeno, aos quais pelo menos um grupo hidroxilo está ligado, estando essencialmente presentes em frutas, vegetais, bebidas e algumas ervas, para as quais contribuem ao nível da cor, sabor e aroma. O seu consumo tem sido associado a uma diminuição do risco de doenças degenerativas em geral, de foro neurológico mas também doenças como o cancro, diabetes, osteoporose e doenças

cardíacas, já que as inúmeras propriedades às quais os (poli)fenóis estão associados vão desde anti-inflamatórias e antioxidantes, até antialérgicas, antivirais, anti proliferativas, anti carcinogénicas e neuroprotetoras. No que diz respeito aos (poli)fenóis presentes em pequenos frutos, estes são reconhecidos pela sua atividade química antioxidante e efeitos neuroprotetores. Nesse sentido, este trabalho teve como principal objetivo a validação do efeito neuroprotetor de metabolitos de amoras digeridas num modelo de neurodegeneração estabelecido a partir de culturas primárias de neurónios de ratinho. A abordagem utilizada contempla o estudo destes compostos após sofrerem uma digestão *in vitro*. Este processo pretende simular as alterações bioquímicas e físico-químicas que ocorrem no trato gastro intestinal superior, permitindo assim uma extrapolação dos metabolitos bioacessíveis ao organismo. No que respeita às amoras, estas são conhecidas por representarem uma rica fonte de (poli)fenóis e a sua popularidade a nível alimentar tem vindo a aumentar, sendo já vários os benefícios documentados resultantes da ingestão deste fruto. No entanto, têm vindo a ser realizadas várias abordagens para análise destes efeitos: *in vivo* / *in vitro*; extrato total / extrato digerido; linhas celulares / células primárias, sendo que nem todos os resultados obtidos apresentam o mesmo significado biológico para a nutrição humana e neuroprotecção.

A cultura de células representa uma ferramenta importante para o estudo das respostas celulares sob condições controladas e quando comparadas entre si, as culturas primárias e linhas celulares divergem em bastantes características, fazendo ressaltar a relevância da utilização de culturas primárias. Estas mimetizam o estado fisiológico das células *in vivo*, o que por sua vez permite a obtenção de dados mais relevantes no que diz respeito aos sistemas vivos. Os neurónios granulares constituem a maior população neuronal do cérebro de mamíferos, sendo por isso frequentemente utilizados como modelos para diferentes estudos. Para além disso, as células cerebelares granulares *in vitro* desenvolvem as características observadas nas mesmas células maduras *in vivo*. Por estas razões, as células cerebelares granulares foram adotadas neste estudo.

Neste trabalho, o estabelecimento de culturas de células cerebelares granulares foi realizado a partir de ratinhos com 7 dias, sendo todas as experiências iniciadas ao 7º dia das células em cultura. Inicialmente, procedeu-se à otimização de alguns dos componentes do meio, nos quais o suplemento B-27 foi testado relativamente à sua composição em moléculas antioxidantes. Após a monitorização diária a nível morfológico (através de microscopia ótica) e a nível da viabilidade celular (através de microscopia de fluorescência por utilização das sondas de iodeto de propídeo e hoechst) durante 11 dias em cultura, a integridade e funcionalidade das células primárias foi confirmada por imunofluorescência através da utilização dos anticorpos para a tirosina hidroxilase e β III-tubulina. Com o objetivo de mimetizar uma condição de neurodegeneração, em que os neurónios se apresentassem

debilitados, o neurotransmissor glutamato foi selecionado como o estímulo químico a introduzir nas células, sendo que após diferentes concentrações serem testadas, se considerou que 90 μM induziam uma percentagem de morte que permitiria detetar uma resposta celular aos extratos anteriormente adicionados. Em seguida, e tendo como finalidade avaliar o efeito neuroprotetor dos metabolitos de amora após sofrerem digestão *in vitro*, diferentes concentrações de extrato compreendidas entre 0 e 1 $\mu\text{g GAE mL}^{-1}$ foram testadas, concentrações essas que se encontram próximas do intervalo de concentrações fisiológicas definidas para compostos fenólicos no plasma humano. Tendo em conta que não foram observadas diferenças significativas entre as diferentes concentrações, nem quaisquer efeitos tóxicos para as células decorrentes das mesmas, foram selecionadas para os estudos subsequentes as concentrações de 0.25, 0.5 e 1 $\mu\text{g GAE mL}^{-1}$. Uma vez testados os extratos digeridos, foram observados resultados de neuroprotecção significativos e semelhantes para as 3 concentrações testadas, quando comparadas com as células expostas apenas ao estímulo tóxico (90 μM glutamato). Numa análise posterior, embora preliminar, foram analisados os complexos mitocondriais da cadeia transportadora de eletrões a nível proteico através da técnica de “western blot”, utilizando uma mistura de anticorpos para os 5 complexos da cadeia de fosforilação oxidativa. Os resultados obtidos permitiram sugerir um maior envolvimento dos complexos I, II e IV em situações de stress relativamente aos restantes. De forma a inferir sobre a possível biogénese mitocondrial, foi iniciado um estudo acerca do número diferencial de cópias de DNA mitocondrial para as diferentes condições testadas. Foram realizados testes preliminares de extração de DNA, quantificação, avaliação da qualidade do mesmo e amplificação por PCR, no qual os primers a serem utilizados foram testados, para posterior aplicação na reação de PCR quantitativo.

Em conclusão, este trabalho permitiu em primeira instância a aquisição do conhecimento necessário ao estabelecimento de culturas primárias de células cerebelares granulares, o estabelecimento de um modelo de neurodegeneração a partir da aplicação de 90 μM de glutamato às células cerebelares granulares em cultura, e a validação do efeito neuroprotetor de diferentes concentrações (0.25, 0.5 e 1 $\mu\text{g GAE mL}^{-1}$) de metabolitos de amoras digeridas, anteriormente verificado num modelo de neurodegeneração em linha celular (SK-N-MC) estabelecido através da aplicação de 300 μM de peróxido de hidrogénio. Para além disso, a análise de proteómica mitocondrial sugeriu a possibilidade de os complexos I, II e IV serem os mais afetados numa situação de stress, havendo uma parcial recuperação do complexo IV na presença dos extratos de amora. No que diz respeito à análise ao DNA, algumas condições inerentes à reação de PCR necessitam ainda de ser otimizadas previamente à execução de PCR quantitativo.

Palavras-chave: doenças neurodegenerativas, células granulares do cerebelo, metabolitos digeridos de amora, (poli)fenóis, mitocôndria

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ABBREVIATIONS

ROS - Reactive Oxygen Species

RNS – Reactive Nitrogen Species

O₂⁻ - Superoxide

OH⁻ - Hydroxyl

H₂O₂ - Hydrogen peroxide

HOCl - Hypochlorous acid

NO - Nitric oxide

ONOO⁻ - Peroxynitrite

ON⁻ - Nitroyl

NO₂ - Nitrogen dioxide

CNS - Central Nervous System

NMDA - N-methyl-D-aspartate (NMDA)

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazole

PTP - Permeability Transition Pore

ATP – Adenosine triphosphate

mtDNA - Mitochondrial DNA

OXPPOS - Oxidative Phosphorylation

ETC - Electron Transport Chain

MOMP – Mitochondrial Outer Membrane Permeabilization

IVD - *In vitro* Digestion

BBB – Blood Brain Barrier

CVD – Cardiovascular Disease

GAE – Gallic Acid Equivalents

RT – Room Temperature

PBS – Phosphate-Buffered Saline

BSA – Bovine Serum Albumine

DAPI - 4',6-diamidino-2-phenylindole

PI – Propidium Iodide

WB – Western Blot

N-PER – Neuronal Protein Extraction Reagent

EDTA – Ethylenediaminetetraacetic acid

SDS-PAGE – Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

PVDF – Polyvinylidene Fluoride

MBA – Membrane Blocking Agent

TBS – Tris-Buffered Saline

HRP – Horseradish Peroxidase

qPCR – Quantitative Polymerase Chain Reaction

ANOVA - Analysis of Variance (ANOVA)

HSD - Honest Significant Difference

PCR – Polimerase Chain Reaction

Cyt b – Cytochrome b

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

TH – Tyrosine Hydroxylase

CYT C – Cytochrome c

1. THEORETICAL FUNDAMENTS

1.1 NEURODEGENERATION

Neurodegeneration corresponds to any pathological condition primarily affecting neurons, which is represented by a large group of neurological disorders with heterogeneous clinical and pathological expressions affecting specific subsets of neurons in specific functional anatomic systems^{1,2}. There are evidences that oxidative stress insult is thought to be one of the main contributing factors to the decrements in cognitive and/or motor performance seen in aging and other neurodegenerative diseases, such as Alzheimer, Parkinson and Amyotrophic Lateral Sclerosis diseases³⁻⁶.

1.1.1 OXIDATIVE STRESS AND GLUTAMATE EXCITOTOXICITY

Glutamate excitotoxicity and oxidative stress are mutually related and represent two important hallmarks of neurodegeneration. The adult brain contains about 10^{11} - 10^{12} neurons², and although it represents only 2% of the body weight, it receives 15% of blood and accounts for 20% of total body oxygen consumption⁷. This, in addition to its high metabolic demand (dependence on oxidative phosphorylation for energy), oxygen consumption and lipid peroxidation content, as well as its low levels of endogenous antioxidants, make neuronal cells highly sensitive to oxidative stress^{2,8,9}. Oxidative stress can be defined as the condition occurring by chemical imbalance between oxidizing agents (reactive oxygen/nitrogen species (ROS/RNS)) and antioxidants (reducing agents and enzymes)^{2,10,11}. The radicals produced due to this imbalance will typically attack lipids, proteins and DNA, damaging cell membranes, enzymes and genetic material¹², leading to many degenerative diseases and aging¹³. ROS are a family of highly reactive small molecules consisting of oxygen free radicals and associated entities that include, among others, superoxide (O_2^-), hydroxyl (OH^\cdot), hydrogen peroxide (H_2O_2), and hypochlorous acid ($HOCl$)^{2,10,14}. RNS in the other hand refer to nitric oxide (NO) and derived molecules, such as peroxynitrite ($ONOO^-$), nitroyl (ON^\cdot) and nitrogen dioxide (NO_2)^{2,14}. These species are formed either during normal metabolism or through external factors, such as x-rays, ultra-violet radiation and pollution¹⁵. In that sense, in order to prevent high levels of ROS/RNS formation, living organisms have complex endogenous antioxidant defense systems (either enzymatic or non-enzymatic)^{12,16}, which are necessary to maintain a physiological balance^{12,14,17}.

Glutamate is the predominant excitatory neurotransmitter, mainly present in the central nervous system (CNS) of the mammalian brain, with up to 40% of all synapses being glutamatergic¹⁸⁻²⁵. Its concentration inside the cells is 5000 to 10000 times greater than outside (at the millimolar level within the vesicle)^{26,27}, is relatively inactive as long as it is intracellular, but extracellularly its action occurs on two cells: presynaptic and postsynaptic

neurons and glial cells inside the brain²⁵. The excitatory responses to this endogenous excitatory amino acid are mediated by a number of pharmacologically and functionally distinct cell-membrane (post-synaptic) receptors: the ionotropic *N*-methyl-*D*-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, as well as the metabotropic ones^{11,19,20,24,25} (fig. 1). Ionotropic receptors are ligand-gated ion channels that open upon the binding of glutamate, and regulate intracellular levels of Na^+ , K^+ and Ca^{2+} , and metabotropic receptors belong to the G-protein coupled receptor superfamily²⁴. Glutamate is both essential and highly toxic at the same time: under physiological conditions, it is released from nerve terminals and is transported primarily to astrocytes, where it is converted to glutamine, which is then stored in glial cells in an inactive form, where glutamate is then regenerated. AMPA receptors are usually co-expressed with NMDA ones at synapses, where they jointly contribute to the process of synaptic plasticity²². Almost every class of glutamate receptors has been implicated in excitotoxic cell death, but it is now accepted that the NMDA receptors play a major role, mainly owing to their Ca^{2+} permeability. At chemical synapses, glutamate is stored in vesicles, until the Mg^{2+} cation is removed, as NMDA receptor is blocked whenever bonded to this ion. At that point, this receptor allows the influx of Ca^{2+} leading to post-synaptic depolarization^{11,22} (fig.1).

Excitotoxicity refers to the neuronal injury and death that occurs as a consequence of excessive accumulation of extracellular glutamate in the synaptic cleft for a prolonged period, a subsequent overstimulation of glutamatergic receptors on the postsynaptic membrane and the associated excessive influx of ions into the cells^{18,19,21,22}. The increase in cytoplasmic Ca^{2+} activates a number of Ca^{2+} dependent enzymes involved in the catabolism of proteins, phospholipids and nucleic acids, increases oxidative stress and may irreversibly damage mitochondria, culminating in neuronal death (fig.1)^{20,28}. Death can occur either by excitotoxic necrosis (involving an uncontrolled influx of Na^+ that results in rapid cell swelling and lysis) or

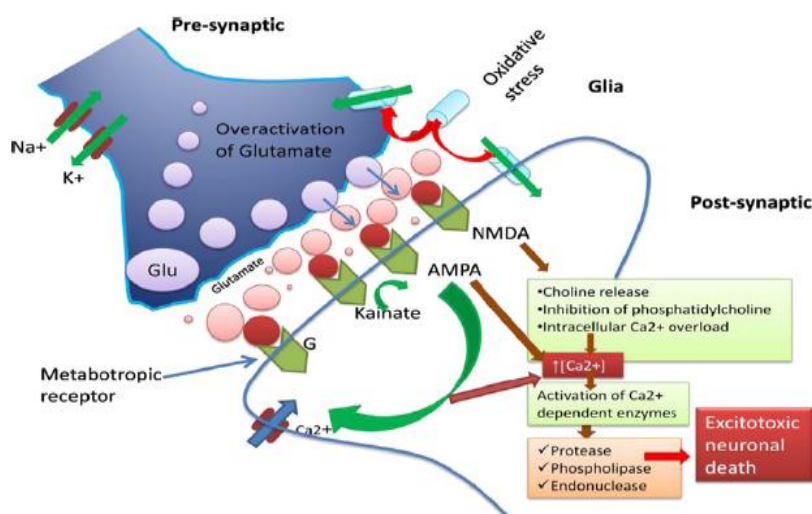


Figure 1 - Schematic representation of the glutamate-induced excitotoxicity mechanism²³.

excitotoxic apoptosis (mediated by excessive Ca^{2+} influx causing the activation of caspases and nuclear chromatin condensation)²⁰. Mitochondrial damage occurs due to the overactivation of NMDA receptors that induce an excessive influx of Ca^{2+} via these ion channels, which in turn attenuates mitochondrial membrane potential, leading at last to the opening of the permeability transition pore (PTP). Through the disruption of mitochondrial potential, excess Ca^{2+} can reduce adenosine triphosphate (ATP) synthesis, rendering the cell more vulnerable to death insults, and making mitochondria to be the primary mediators of cell death caused by excitotoxicity²⁴. In general, the common mechanisms of excitotoxicity can be defined as free radical generation and inhibition of antioxidant enzymes, mitochondrial energy enzymes and glutamate transporters¹⁹. Therefore, an evident relationship can be established, as excitotoxicity leads to oxidative stress which not only causes neuronal dysfunction and cell death, but also triggers excitotoxicity itself. Excitotoxicity happens by impairing glutamate removal and activating microglia, that contain abundant stores of glutamate, leading at the end to age-related chronic neurodegenerative diseases^{19,25,29}.

1.1.2 MITOCHONDRIA FUNCTION AND BIOGENESIS

As critical organelles in cell survival, by their participation in a number of cellular functions such as modulation of: cellular calcium homeostasis; free radicals; apoptosis; amino acid biosynthesis; fatty acid oxidation; steroid metabolism; and maintenance of cellular energy reserves through ATP production, mitochondria play an essential role in eukaryotic cells^{2,8,11,30}. It has been estimated that about 90% of mammalian oxygen consumption is at mitochondria, which primarily serves to ATP synthesis, making mitochondria particularly important in neurons, owing to their high demands for energy already mentioned^{2,31}. Mitochondria suffer continuously changes on their morphology, length, size, number, function and distribution, through a delicate balance between fission and fusion processes^{29,30}. Fission and fusion are antagonistic processes that predominate under different conditions to adapt mitochondrial morphology and dynamics to the bioenergetic requirements of the cells³². During fission, mitochondrial DNA (mtDNA) and other critical mitochondrial contents are redistributed to the daughter mitochondria by being transformed into isolated, small and round organelles. These fragmented mitochondria are frequently found in resting cells, when high respiratory activity is not required, contributing to the maintenance of bioenergetic capacity³³. Fusion on the other hand allows the mixture and exchange of mitochondrial contents (including mtDNA and its encoded proteins), which will then enable individual mitochondrion to rapidly replenish its possible lost components. Fused mitochondria are frequently found in respiratory active cells, when optimal mitochondrial function is needed, either due to the absence of a functional respiratory chain or due to a significant reduction of

cellular ATP levels, as fusion allows constituents of the respiratory machinery to cooperate most efficiently^{32,33}. However, an altered balance between fission and fusion may affect the removal of damaged mtDNA and thus contributes to the accumulation of mutant mtDNA³³. Although the biogenesis of mitochondria depends on an intricate cross talk between mitochondrial and nuclear genomes, mitochondria are the only organelles in animal cells (besides nucleus) that contain their own separate DNA³⁴, which by complementarity with the nuclear genome encodes genes involved in local mitochondrial protein synthesis and protein subunits of the oxidative phosphorylation (OXPHOS) complexes^{21,35}. These multiprotein complexes constitute the mitochondrial electron transport chain (ETC), which is composed of a series of electron carriers that are spatially arranged according to their redox potentials and organized into four complexes (fig.2), named NADH:ubiquinone oxireductase (complex I), succinate:ubiquinone oxireductase (complex II), ubiquinol-cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV), as well as the electron carriers ubiquinone and cytochrome c and the H⁺-ATP synthase complex^{30,36}.

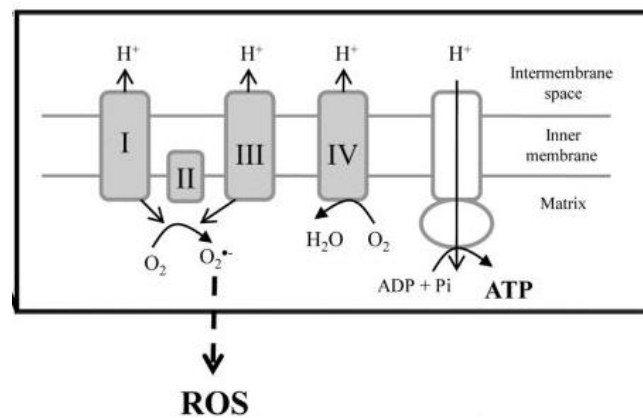


Figure 2 - Schematic representation of the mitochondria electron transport chain. Electro leak from the ETC results in the production of superoxide (O₂^{•-}), which is quickly and efficiently converted to H₂O₂, which is in turn the principal cellular mediator of oxidative stress because of its relative stability and membrane permeability³⁸.

Knowing that mitochondrial oxidative phosphorylation is the major source of ROS generation^{7,14,37}, this mechanism is believed to be important in the aging process as well as in the pathogenesis of neurodegenerative diseases, considering complex I the major physiologically and pathologically relevant ROS generating site³⁸. The formation of ROS occurs when unpaired electrons escape the electron transport chain and react with molecular oxygen, generating superoxide (fig.2), which can in turn lead to oxidative damage of mitochondrial protein, membranes and DNA³⁴, impairing the ability of mitochondria to synthesize ATP and to carry out their wide range of metabolic functions (fig. 3)³⁹. Mitochondrial oxidative damage can also increase the tendency of mitochondria to release intermembrane space proteins such as cytochrome c to the cytosol by mitochondrial outer membrane permeabilization (MOMP) and thereby activate the cell's apoptotic machinery (fig.

3). In addition, mitochondrial ROS production leads to induction of the PTP, which renders the inner membrane permeable to small molecules in certain situations³⁹. As mentioned before, mitochondria are essential for neuronal function because the limited glycolytic capacity of neurons makes them highly dependent on aerobic OXPHOS for their energetic needs⁷. However, oxidative stress directly leads to mitochondrial dysfunction¹⁴, which means that any mitochondrial injury can have severe consequences for neuronal function and survival (fig. 3)²¹. In fact, mitochondrial dysfunction (associated with a functional decline) is becoming appreciated as a unifying characteristic of diverse degenerative pathologies⁴⁰, contributing to the aging process, in which an activity reduction of any ETC component is detected, and is in turn associated with increased mitochondrial biogenesis.

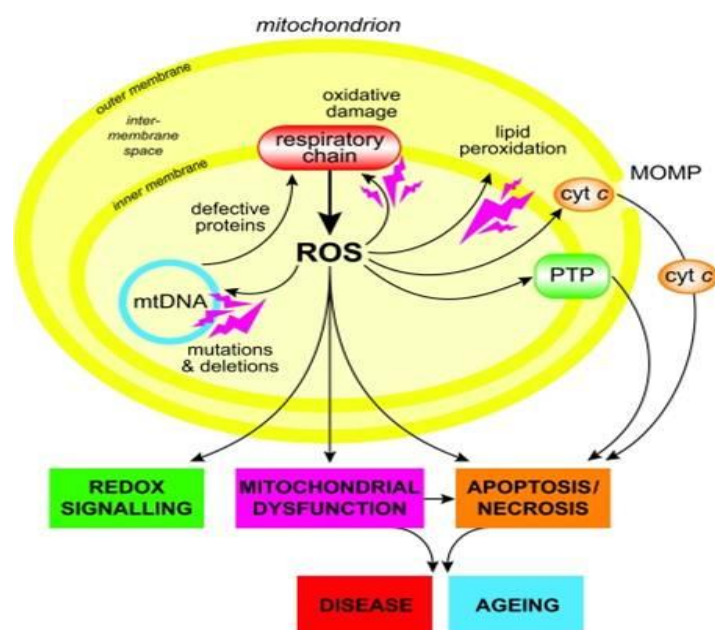


Figure 3 - Schematic representation of mitochondrial oxidative damage and its consequences³⁹.

There is information stating that small, physiological increases in ROS are required for normal increases in mitochondrial biogenesis and moreover that this process may represent an attempt by cells either to increase their aerobic set point, or to maintain a pre-existing aerobic set point in the face of declining mitochondrial function. This relationship is no longer observed either when the decline keeps further to the point that mitochondrial biogenesis can no longer compensate functional declines or by the administration of certain compounds, that may actually serve to disrupt this delicate physiologic feedback loop³⁴. Accordingly, measurement of mitochondrial synthesis is considered to be the correct approach to assess mitochondrial biogenesis, at protein or DNA level (instead of the frequently used assessments of signaling or content)³⁷.

There is no doubt that mitochondrial abnormalities and dysfunction are involved in neurodegenerative diseases, contributing to the aging process³⁷. However, it is still a matter

of debate whether or not such abnormalities are a cause or merely a consequence among many of the diseases²¹.

1.2 BERRIES AND (POLY)PHENOLS

Quality of life at older age is strongly linked to the preservation of cognitive function that can be influenced by numerous factors and one of the most obvious, but yet under-recognized, being the role of nutrition⁴¹.

(Poly)phenols represent a wide variety of compounds essentially found in fruits, vegetables, beverages and some herbs, which are responsible for their colour, flavour, aroma and appearance, and whose primary function is protection of plants against ROS produced during photosynthesis and against herbivores⁴¹⁻⁴⁴. Characteristically, they have at least one aromatic hydrocarbon ring attached with one or more hydroxyl groups, are mostly found as sugar conjugated⁴¹, have ROS scavenging properties and seem to be important in the prevention of different diseases associated with oxidative stress, such as neurodegenerative and cardiovascular diseases and cancer⁴². Although (poly)phenols are greatly abundant in our diet, they are poorly absorbed from the intestine, highly metabolized and rapidly eliminated, therefore their metabolites are the bioactive compounds within the body^{42,44}. (Poly)phenols can be classified into two main categories, namely flavonoids and non-flavonoids. Flavonoids include flavonols (eg.: quercetin, kaempferol and myricetin), flavones (eg.: apigenin and luteolin), flavan-3-ols (eg.: catechin and condensed tannins (proanthocyanidins), flavanones (eg.: naringenin, eriodictyol and hesperitin), anthocyanins (eg.: callistephin, chrysanthemin and peonidin-3-O-glucoside) and isoflavonoids. Non-flavonoids on the other hand include phenolic acid (eg.: hidroxybenzoates), lignans and stilbenes (resveratrol)^{13,41,44,45}.

Consumption of fruits and vegetables has been associated with a decreased risk for certain degenerative diseases, not only for neurological ones, but also for cancer, stroke, heart disease, diabetes mellitus and osteoporosis, due to the (poly)phenols' anti-inflammatory, antioxidant, antiallergic, antiviral, antiproliferative, anticarcinogenic and neuroprotective and thus "anti-aging" health effects^{4,46}.

Fruits, and specially berries phytochemicals are well known for their chemical antioxidant activity⁴⁷ and neuroprotective effects⁴⁸. In addition to their attractive sensorial attributes (color, aroma and flavor), berries also display a high nutritional quality, as they are low in calories and a good source of vitamin C, dietary fiber and minerals, containing high levels of natural polyphenol contents, from which anthocyanins are the most abundant^{49,50}. They constitute a group of over 500 compounds that provide the red to purple and blue pigments of many vegetables and fruits¹³, are reactive compounds and readily degrade or react with other constituents in mixtures to form colorless or brown compounds⁵¹. Therefore, the dietary

choice (different berries) has a substantial impact on the amount of anthocyanins and other (poly)phenols consumed, and consequently, the amount of health benefits they are associated with⁵¹.

1.2.2 BLACKBERRIES DIGESTED METABOLITES - *IN VITRO* DIGESTION (IVD)

When applying fruits and other food components to *in vitro* studies, it is expected that they have been previously gone through digestion, absorption and metabolization processes. Therefore, in this sort of studies it is used an extrapolation of the bioaccessible metabolites, which refers to the amount of potentially absorbable form of ingredients into the body⁴¹.

In vitro digestion models are widely used to study the structural changes, digestibility, absorption and release of food components under simulated gastrointestinal conditions, mimicking as much as possible the human digestion⁵², in other words, it is a procedure that simulates the physicochemical and biochemical changes that occur in the upper gastrointestinal tract. This is easily reproducible, although requires adjustments according to the type of food being digested. For the present work, the main purpose of performing *in vitro* digestion of blackberry fruits is essentially trying to obtain the metabolites that will possibly reach blood stream and consequently the blood brain barrier (BBB) and exert any neuroprotective effect. This process is particularly important, as it was previously demonstrated that the original undigested berry extract (specifically blackberry), although exhibiting a significant chemical antioxidant capacity *in vitro*, was not able to decrease ROS production in cells submitted to an oxidative stress. On the contrary, digested berry extracts potentiated the ROS decrease³.

1.2.3 BLACKBERRIES AND NEUROPROTECTION

Blackberry fruits are well known to be a rich source of (poly)phenols (mainly anthocyanins and ellagitannins)¹³ and to exhibit high natural chemical antioxidant capacity, which is one of the major reasons for their increasing popularity in the human diet^{3,48,53}. There are several positive and beneficial documented effects from blackberries ingestion, such as age-related changes and prevention of age-related neurodegenerative diseases¹³, management of inflammation by suppressing pro-inflammatory cytokines and cancer prevention, as it was shown potential in inhibiting the growth of human colon cancer¹⁵.

Dissecting the information obtained from the studies available, its different conditions in terms of type of the assay (*in vitro* or *in vivo*), type of berries, way of administration (digested or non digested), and targets (either animals or humans) becomes evident, there is a huge and variable pool of results regarding berries and its benefits. However, not all have the same biological significance for human nutrition and neuroprotection. *In vitro* studies of neuroblastoma cell line subjected to an H₂O₂ stress and wild blackberries digested extract

resulted in recovery of membrane potential and cell membrane integrity, ensuring cell viability, constituting by then promising sources of metabolites with neuroprotection capabilities⁴⁸. In a similar study, having the same neuroblastoma cells subjected to the same stress, blackberry digested metabolites were able to maintain cell membrane integrity, protecting neurons from death, which in that case was not necessarily associated to enhanced antioxidant capacity³. In addition to those, *in vivo* studies which use animals (usually rodents) as the study group can also be found. Their advantage is that, apart from all the chemical and biochemical analysis made to cells, they allow the observation of differences in motor and cognitive functions. A number of these studies have documented that rodent diet supplementation with berries is effective in reducing not only oxidative stress but also neuronal, behavioral and cognitive defects associated with aging, inflammation and ischemia⁵⁴. Other similar *in vivo* studies, with certain extracts supplementation, have demonstrated retarded age-related decrements, improved performance on motor tests, improved working memory performance and increases in several indices of neuronal signaling⁵⁵. When rats had a long-term intake of blackberry extracts (anthocyanins and ellagitannins), the markers for the oxidative status in some tissues are changed and the expression and activities of antioxidant enzyme are altered when compared with animals that did not receive (poly)phenols in their diet¹⁶. A number of other *in vivo* studies applied not only to animals but also to humans have been made in order to investigate not only the bioavailability of anthocyanins⁵¹, but also the berries impact on cardiovascular health⁴⁹. Amongst other conclusions, volunteers showed a low risk of cardiovascular disease (CVD) during berries enriched diet (by the maintenance of normal vascular function and blood pressure), a decrease on an inflammation marker, and a decrease of lipid peroxidation in smokers.

From all these assays, it becomes evident that berries, and specifically blackberries have a distinguished role on human health, mainly proved in terms of age-related diseases and neurodegeneration. In that sense, the usage of this berry fruit as this study object becomes urgent and relevant.

1.3 NEURODEGENERATION CELL MODEL

Cell culture offers a mean to study cellular responses *in vivo* under controlled conditions and has been increasingly used as an investigation tool⁵⁶. Comparing primary cultures to cell lines, numerous characteristics diverge, standing out the importance of using primary neurons in this work. Regarding cell lines, although they can be subcultured infinitely, have minimal variability and are easy to work with, they are subjected to gross mutations and chromosomal abnormalities, which provides poor indicators of normal cell phenotype and progression of early-stage disease⁵⁷. In general, primary cells are formed from cells that

survive the disaggregation process, attach to the cell culture support and proliferate, being morphologically similar to the parent tissue and physiologically similar to *in vivo* cells. Although they have issues of limited cell accessibility and comparability, difficulty with cell isolation/purification, quality assurance and consistency, and contamination risks, primary cells more closely mimic the physiological state of cells *in vivo*, which in turn allows the generation of more relevant data representing living systems^{56,57}.

When comparing these two cell types in terms of literature, it easily becomes evident that studies and investigations concerning beneficial effects of nutrition, developed on cell lines is more common. In the case of cell lines such as SK-N-MC^{3,48}, SH-SY-5Y (human neuroblastomas)⁵⁴, HT-29 (human colon tumor)⁵⁸ and MCF-7 (human breast tumor)⁵³, a plenty of studies have been developed, usually using a stress inducer, like H₂O₂⁵⁹ that proceeds a previous treatment with different (poly)phenols extracts. Regarding primary neurons, either hippocampal⁴⁷, cerebellar⁶⁰ or others, few studies have been done. Although these cells are used for a plenty of different studies as well, also using a stress inducer^{21,23,28,29,61-63}, they have not often been applied specifically to test berries neurological preventive effects.

With the increasing evidence that neurodegeneration is related to oxidative stress, which in turn can be triggered by excitotoxicity, exposure of cells to glutamate can be used as a valuable model of the mechanisms of oxidative injury in the CNS, and may prove very useful in the elucidation of protective strategies in neurodegenerative diseases associated with oxidative damage or stress. Taking that in consideration, glutamate has been used extensively as an inducer of oxidative stress in different *in vitro* models^{28,61-63}, namely those from cerebellar granule cells⁶⁴.

The great majority of cerebellar neurons, both in cerebellum and whole brain, are granular, which constitute the largest homogeneous neuronal population of the mammalian brain, being often used as a model system for the study of neuronal development, function and pathology, including the analysis of activity-dependent survival/apoptosis of neurons and the mechanisms of neuroprotection. In addition, when cultured *in vitro*, cerebellar granule cells develop characteristics of mature cerebellar granule cells seen *in vivo*, such as an extensive neuritic network, expression of excitatory amino acid receptors and production and release of glutamate⁶⁴.

2. OBJECTIVES

Since few years that both scientific and non-scientific societies have been assisting to an increased interest in berries, in particular due to their highly advertised benefits, with great impact on several areas of the human health. In that sense, nutrition can be considered as an emerging preventive solution for several disorders, mainly neurodegenerative ones.

Neurodegeneration is in intimate association with both excitotoxicity and oxidative stress. Several preventive approaches have been studied, namely with regards to berries, which have been referred for their highly neuroprotective potential but still, lacking to be tested on primary cultures of neurons. Therefore, this study renders to be necessary, as it will hopefully bring up new information on how all these mentioned factors work together to generate health and/or disease. In that sense, some main goals for this work were settled:

- To establish a primary culture from mice granular cerebellar cells and to learn about their behavior and development along the days in culture;
- To determine if the neuroprotective effects previously verified on a neuronal cell line, are present in a model of mice primary culture of neurons, which meets the present goals of the assay. So far, in DSB Lab there used to be an implemented neurodegeneration cell model, developed with SK-N-MC, which is a neuroblastoma continuous cell line, established in culture from human metastatic neuroblastoma tissue⁶⁵. However, for the purpose of this work, a more physiological model based on mice cerebellar neurons was used instead. The main characteristics of this model make it extensively characterized and used for *in vitro* studies⁶⁴;
- To evaluate mitochondrial population as an indicative of mitochondrial biogenesis;
- To understand how the overall expression of mitochondrial complexes occurs within different culture conditions, to realize if any of them is over or underexpressed, and to determine the main complex(es) involved in oxidative stress/excitotoxicity processes and by then, also determine a possible pathway for neuroprotection;

In order to achieve these goals, four main tasks were proposed:

- Establishment of primary culture of neurons from seven day-old mice;
- Establishment of a neurodegeneration model based on an oxidative stress by testing different times and concentrations of exposure to glutamate;
- Evaluation of blackberries digested metabolites possible neuroprotective effects on cells by exposing primary neurons to these metabolites;
- Assessment of mitochondria biogenesis by protein analysis, to identify whether digested metabolites induce it and how it may alter cellular metabolism and enforce oxidative phosphorylation.

3. MATERIALS AND METHODS

3.1 PLANT MATERIAL, EXTRACT PREPARATION AND *IN VITRO* DIGESTION

Fruits of the commercial blackberries cultivar Apache (*Rubus* L. subgenus *Rubus* Watson) produced in Fataca experimental field (Odemira, Portugal) were previously obtained, its extract prepared and stored in DSBLab, as described before⁴⁸. In addition to that, these hydroethanolic extracts went through phytochemical alterations occurring during digestion, which were done in collaboration with James Hutton Institute and mimicked using the IVD model previously described³. Digested samples were dried and stored at -20°C until use.

3.2 CELL CULTURE

3.2.1 CEREBELLAR CELL CULTURE

Primary cultures of cerebellar granule cells were prepared according to the already described method⁶⁰. Briefly, cells were isolated from the cerebella of 7-day-old BALB-C mice and went through a manual disruption process, where cerebella was cut with a razor in parallel and perpendicular movements, after which the tissue was centrifuged 1 min. at 200 g. The following process was a chemical disruption with a mild trypsinization (200 mg L⁻¹, Sigma) of the tissue, followed by trituration in a DNase solution (10 mg mL⁻¹; Sigma), simultaneously with its constant mixing at a 37°C water bath. Trypsinization was then inhibited and a new centrifugation for 10 min. at 200 g was made. As the last mechanism to isolate cells from the original tissue, it was minced with a puncture needle, and another centrifugation of 5 min. at 200 g was made. After cell counting, cells were resuspended (0.5 x 10⁶ cells mL⁻¹) and cultured in Neurobasal Medium (Gibco) supplemented with 2% (v/v) B-27 without antioxidants (alpha tocopherol acetate, alpha-tocopherol, catalase, superoxide dismutase and glutathione (Gibco)) and 2% (v/v) KCl (20mM, Sigma), and containing 0.25% (v/v) glutamine (200 mM, Sigma) and 0.48% (v/v) penicillin-streptomycin (5000 U mL⁻¹, Gibco) solution. Cells were lastly cultured in plates or flasks coated with poly-D-lysine (50 µg mL⁻¹, Sigma) and maintained in a humidified atmosphere of 7% CO₂ at 37 °C. Half of the media (either 500 µL or 5 mL) was removed 48 h after inoculation, maintained at 37°C, and cytosine arabinoside (20 µM, Sigma) was added to prevent glia cell proliferation, with all the experiments being performed on days 7-11 in culture (fig. 4). The removed media was introduced back in the culture at day 7, and used either for medium renewal or for the introduction of any new compound to the culture. Most of the experiences were performed on 24-well plates covered with coverslips and coated with poly-D-lysine.

3.2.2 B-27 SUPPLEMENTATION TEST

Primary cultures were established with B-27 either with or without antioxidants. Moreover, to analyze if antioxidants from B-27 could interfere with blackberry digested extract effect, different concentrations of digested extract were tested. In order to do that, at day 7, half of the media was removed and the extracts added, which had been previously homogenized in the preserved medium. Concentrations tested were: 0.25, 0.5 and 1 μg gallic acid equivalents (GAE) mL^{-1} . Cells were incubated for 24 h, morphology was monitored by optical microscopy and cell viability was determined.

3.2.3 IMMUNOFLUORESCENCE

The integrity and functionality of primary cultures of cerebellar neurons were confirmed by immunofluorescence of cells cultured for 7 days, according to the protocol previously described⁶⁶. Briefly, cells were fixed in a solution of 4% paraformaldehyde plus 4% sucrose for 20 min. at room temperature (RT), washed twice in phosphate-buffered saline (PBS) and then blocked and permeabilized with PBS containing 3% bovine serum albumin (BSA, Sigma) and 0.1% Triton X-100 (Merck) for 20 min. at RT. Cells were incubated with anti-tyrosine hydroxylase (rabbit polyclonal, 1:200, Santa Cruz Biotechnology Inc., Nr. Sc-14007) and anti-Beta III tubulin (mouse monoclonal, 1:200, Millipore, Nr. MAB1637) for 2 h at RT. Immunolabeling was visualized by incubation with AlexaFluor 594-conjugated goat anti-rabbit IgG (1:500, Invitrogen) and AlexaFluor 488-conjugated goat anti-mouse IgG (1:500, Abcam). After two washes in PBS, immunolabeled cells were mounted and counstained with ProLong[®] Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). After drying, cells were observed and photographed under a computer-driven digital camera (Leica DFC340 FX, Heerbrugg, Germany) on an upright microscope (Leica DMRB microscope, Wetzlar, Germany) using filter cubes with a bandpass of 515-560 nm, 450-490 nm and 355-425 nm of wavelength, for tyrosine hydroxylase, beta III tubulin and DAPI, respectively (fig. 4).

3.3 CELL VIABILITY

Cell viability was assessed in all cases by fluorescence microscopy through Propidium Iodide (PI) and Hoechst 33342 staining, as previously described⁶⁰. Cells were incubated with Hoechst (2 μM , Sigma) and PI (1 μg mL^{-1} , Invitrogen) for 10 min. at 37°C. Coverslips were then removed from each well and monitored on slides. Fluorescent images were taken with a computer-driven digital camera (Leica DFC340 FX, Heerbrugg, Germany) on an upright microscope (Leica DMRB microscope, Wetzlar, Germany) using filter cubes with a bandpass of 355-425 nm and 515-560 nm of wavelength, for Hoechst and PI, respectively.

For image analysis of cell viability, 3 micrographs per coverslip were taken at a 40 X magnification, meaning that for each experiment, 9 micrographs per condition were evaluated. Image analysis was performed with ImageJ 1.47. Brightfield images for morphology evaluation were taken at 20 and 40 X magnification, with a computer driven digital camera (Nikon Digital Sight DS-Fi1) on an inverted microscope (Nikon Eclipse TE300).

3.4 GLUTAMATE OPTIMIZATION

To investigate the appropriate glutamate concentrations (that allow a range of death percentage enough to make the cells able to respond to the further stimulus) for the establishment of the neurodegeneration cell model, at day 8 in culture cells were exposed to different concentrations of glutamate solution (Sigma), from 10-100 μM . Controls were cells without glutamate treatment, and processed in parallel. Cells were then incubated for 24 h, morphology was evaluated and viability determined as previously described at 3.3 topic (fig. 4).

3.5 CYTOTOXICITY EVALUATION

To evaluate extracts cytotoxicity, dried digested fractions were resuspended in cell medium. At day 7 in culture, 500 μL of culture medium was removed and primary neurons were incubated with different concentrations of these fractions, in the range of 0-1 $\mu\text{g GAE mL}^{-1}$ medium. Cells were then incubated for 24h, morphology was evaluated and viability determined as previously described at 3.3 topic (fig. 4).

3.6 NEUROPROTECTIVE EVALUATION

To determine the neuroprotective effects of blackberries digested metabolites, at day 7 in culture, non-toxic concentrations (0.25, 0.5 and 1 $\mu\text{g GAE mL}^{-1}$) of blackberry dried fractions were resuspended in 500 μL of cell medium (previously removed at day 2 and maintained at 37°C) and added to the cells, after a previous removal of 500 μL of the medium from each well. Cells were then incubated for 24 h and morphology evaluated. At day 8 in culture, 90 μM of glutamate was added to the respective wells. Cells were incubated for 24 h, morphology was evaluated and viability determined as previously described at 3.3 topic (fig. 4).

3.7 WESTERN BLOT (WB) ANALYSIS

3.7.1 PROTEIN EXTRACTION

Neurons cultured on 25 cm^2 t-flasks were submitted to protein extraction on day 9 in culture through the Neuronal Protein Extraction Reagent (N-PER, Pierce) supplemented with an

ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (1% (v/v), Calbiochem), according to the manufacturer's instructions. Briefly, cells were lysed with the previous solution on ice for 5 min. following a 10 min. centrifugation at 10.000 g, after which the supernatant was collected and stored at -20°C until being used. Protein quantification was then processed using modified Lowry's method.

3.7.2 ELECTROPHORESIS AND ELECTROTRANSFER

Twenty µg of protein, as well as the prestained protein marker VI (Applichem) and the rat heart mitochondria extract as positive control (Mitosciences - Abcam) were subjected to a sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) in 10% (w/v) and 1.5 mm thickness acrylamide gel, at 200 V for approximately 40 min. The electrophoresis buffer was composed by 25 mM Tris-base, 190 mM glycine and 0.1% (w/v) SDS⁶⁷.

The electrotransfer of proteins occurred according to the previously described method⁶⁸. After SDS-PAGE, gel was soaked in transfer buffer composed by 12 mM Tris-base, 96 mM glycine, 0.1% (w/v) SDS and 20% (v/v) methanol at pH 8.3. A polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences) was also incubated in transfer buffer for 5 min. after a 1 min. pre-incubation in methanol. Both gel and membrane were assembled in the transfer cell (Trans-Blot electrophoretic transfer cell, BioRad) containing transfer buffer, and transference was carried out at approximately 70 V for 2 h at 4°C.

3.7.3 IMMUNODETECTION

The expression of mitochondrial OXPHOS system components was assessed by using the MitoProfile[®] Total OXPHOS Rodent WB primary antibody cocktail (MS604, Mitosciences - Abcam) targeting specific subunits from mitochondrial complexes I, II, III, IV and V, according to manufacturer's instructions and other previously described data^{69,70}.

After transfer, membrane was dried at RT and blocked with 5% (w/v) membrane blocking agent (MBA, GE Healthcare) in Tris-buffered saline (TBS) containing 0.01% (v/v) Tween20[®] (Sigma) (TBST), for 1 h at RT. Membrane was then incubated with primary antibody (1:250) and left overnight at 4°C, after which it was washed three times in TBST for 5 min. each, and incubated with secondary antibody (stabilized goat anti-mouse horseradish peroxidase (HRP) – conjugated, 1:2500, Thermo Fisher Scientific Inc.) for 2 h at RT. Rabbit anti-actin primary antibody (sc-1616-R, 1:1000, Santa Cruz Biotechnology) was used as loading control, along with goat anti-rabbit HRP – conjugated secondary antibody (1:300, Millipore). All antibodies were diluted in 5% MBA. Antibody detection was performed with a chemiluminescent substrate (FemtoMax Super Sensitive Chemiluminescent HRP Substrate,

Rockland Inc.) and membrane images were acquired in the Molecular Imager Chemidoc XRS (Quantity One[®] software 4.6.6, BioRad).

3.8 POLIMERASE CHAIN REACTION (PCR)

Preliminary tests on PCR with extracted DNA samples were executed, as they represent the necessary analysis preceding a quantitative PCR reaction. In order to do that, genomic DNA was extracted from primary neurons cultured under control conditions (0 µg GAE mL⁻¹ and 0 µM glutamate) using the High Pure PCR Template Preparation Kit (Roche Diagnostics), according to the manufacturer's instructions, and further stored at -80 °C until use. For the assessment of DNA quality and yield, samples were analyzed with a NanoDrop 2000c spectrophotometer (Thermo Scientific) and DNA integrity and contamination were determined by DNA visualization in a 1% agarose gel, stained with ethidium bromide.

Potential DNA contamination and primers efficiency were tested through PCR, which was performed using specific forward and reverse primers designed for the mitochondrial cytochrome b (cyt b) gene (forward: 5'-TTC ATG TCG GAC GAG GCT T-3' and reverse: 5'-TCC TCA TGG AAG GAC GTA GG-3') and for the nuclear glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (forward: 5'-CCT TCA TTG ACC TCA ACT ACA T-3' and reverse: 5'-CCA AAG TTG TCA TGG ATG ACC-3')⁷¹. The PCR reaction contained 2 µL input (20-100 ng DNA), 0.625 U Taq DNA Polymerase (Fermentas), 1.25 µL of each forward and reverse primer, 2 mM MgCl₂, 0.2 mM of each dNTP (Invitrogen), 75 mM Tris-HCl (pH 8.8 at 25 °C), 20 mM (NH₄)₂SO₄ and 0.01% (v/v) Tween 20. The following program was applied: 1 cycle of 3 min. at 94°C for denaturation, followed by 41 cycles (45 sec. at 94 °C for denaturation, 30 sec. at 60 °C for annealing, 90 sec. at 72 °C for extension) and a final 10 min. extension at 72 °C. PCR products previously obtained were visualized in a 2% agarose gel stained with ethidium bromide. Both gel images were acquired in the Molecular Imager Chemidoc XRS (Quantity One[®] software 4.6.6, BioRad).

3.9 STATISTICAL ANALYSIS

All the results reported are the averages of at least three independent experiments, which data was statistically evaluated either by analysis of variance (ANOVA) – Tukey honest significant difference (HSD) test, or by Paired t-Test, using SigmaStat 3.10 software (Systat Software Inc.).

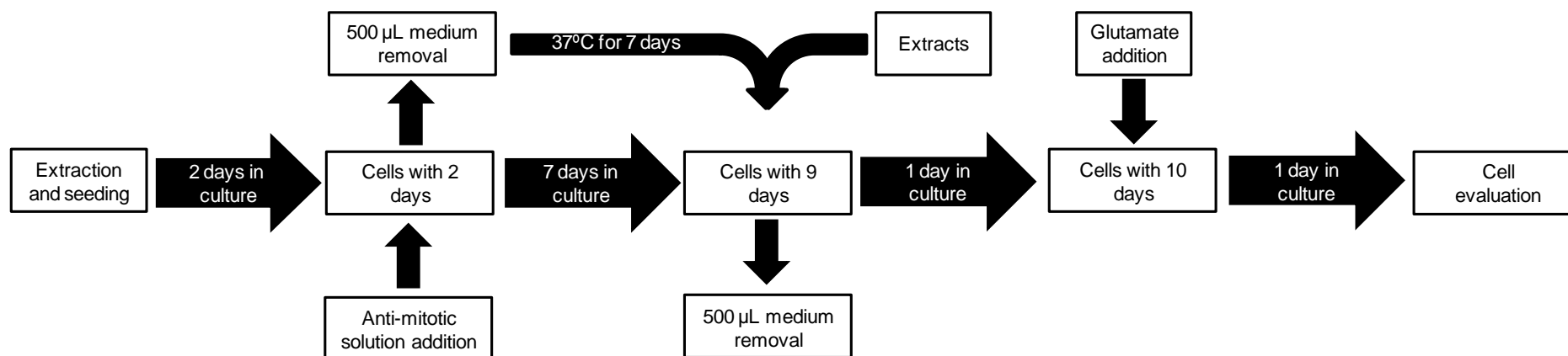


Figure 4 - Overall flowchart of the main steps involved on cerebellar granule cells culture, occurring during the four tasks proposed.

4. RESULTS AND DISCUSSION

4.1 ESTABLISHMENT OF PRIMARY CULTURE OF NEURONS

The initial stage of this work was the establishment of primary culture of neurons from mice cerebellum. The choice of this brain's region was related to its high content on neuronal cells that increases the probability of getting viable neurons for cell culture. The use of 7 day-old mice is an important issue and using older animals is not recommended, as cells will start to extend axons and form dendrites and synaptic connections, rendering them more susceptible to damage during the purification process⁶⁴.

B-27 is recommended to be used as a supplement within Neurobasal media. This supplement has a diversified composition, presenting proteins (such as BSA and insulin), vitamins (such as vitamin A and tocopherols), antioxidants (alpha tocopherol acetate, alpha-tocopherol, catalase, superoxide dismutase and glutathione) and other components (such as some hormones and D-galactose), however for our study raises some questions related to the antioxidant and protein contents:

- The high content on antioxidants mentioned before (alpha tocopherol acetate, alpha-tocopherol, catalase, superoxide dismutase and glutathione) could interfere and mask any possible effects of blackberries digested metabolites on cells, therefore we have decided to test the commercially available B-27 supplement without antioxidants, and determine whether it could effectively replace B-27 with antioxidants without affecting cell viability. According to the results obtained, for a control situation in which cells were cultured in medium containing B-27 with and without antioxidants in the absence of blackberries digested extracts ($0 \mu\text{g GAE mL}^{-1}$), no statistical significance was observed on cell viability between both conditions. Moreover, when increasing concentrations of blackberries digested metabolites (0.25 , 0.5 and $1 \mu\text{g GAE mL}^{-1}$) were added to the culture medium in the presence of both types of B-27, the same statistical indifference was detected between the two medias tested, for each extract concentration (data not shown). Therefore, we decided to culture cells in B-27 without antioxidants supplemented medium;
- Concerning protein content of B-27 supplement, and since protein interaction with (poly)phenols further added to the media was a concern, we then considered to test different percentages of B-27 in order to minimize it as much as possible. However, because its percentage within the media is already low, we have considered that the interference between protein and (poly)phenols would be minimal and decided to maintain the original recommended B-27 concentration as 2% of the media.

After the media parameters were defined, cells were monitored in culture for 11 days in order to understand and evaluate their growing state and progression throughout the time in

culture. Cell viability was determined for each day in culture, and according to the results obtained, the percentage of viable cells did not change considerably over days (fig. 5), meaning that cells in culture are usable for experiences during that time. Microscopic morphological analysis was also performed and by the results achieved, major differences were evident from day 1 to day 3 in culture, from which they develop their cellular bodies and elongate dendrites, being considered ready to use for experiences after day 7 (fig. 6).

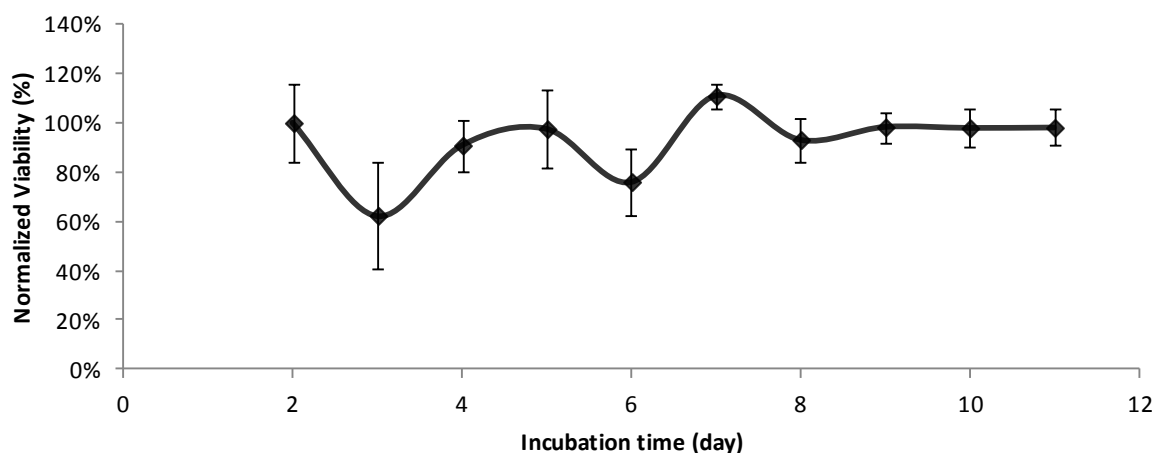


Figure 5 - Viability of primary neurons monitored from day 1 to 11 in culture, through PI/Hoechst staining.

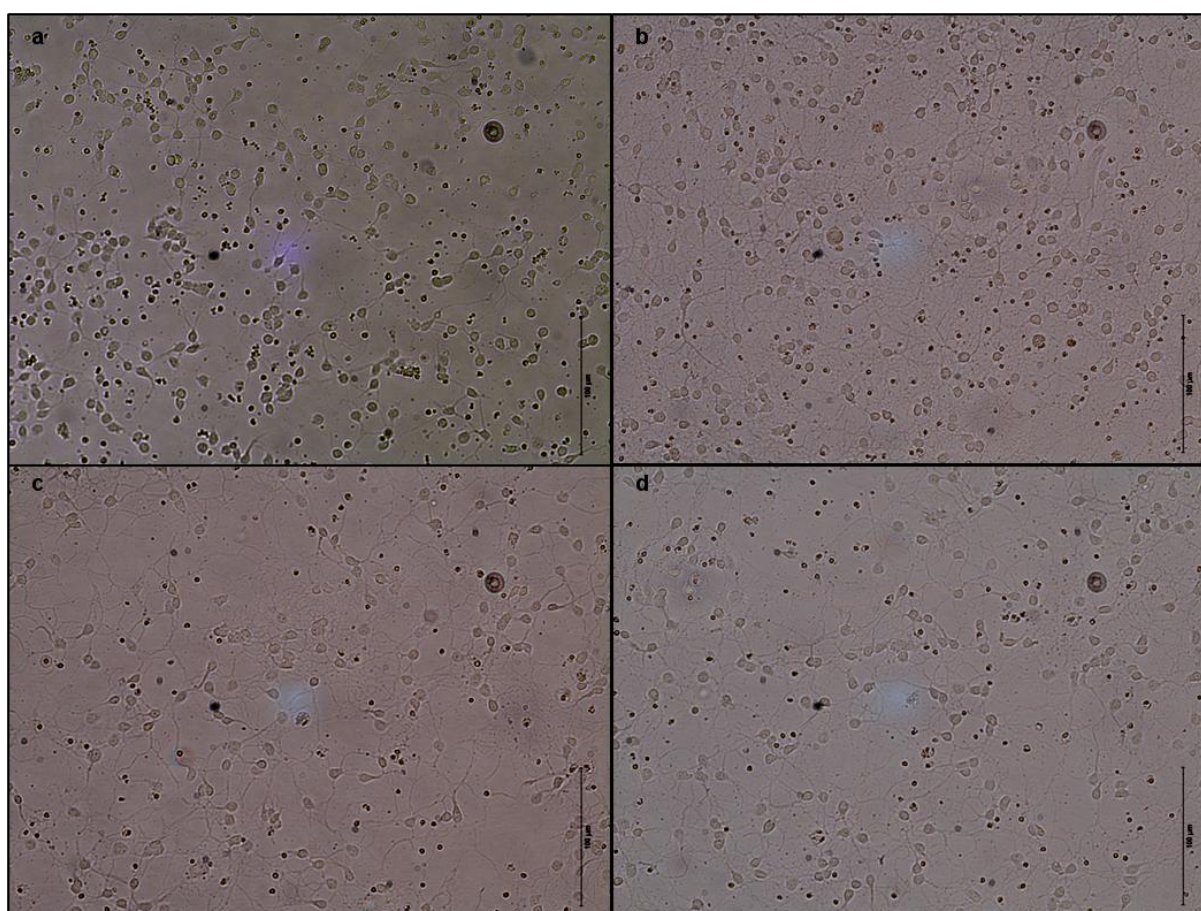


Figure 6 (see legend on the next page)

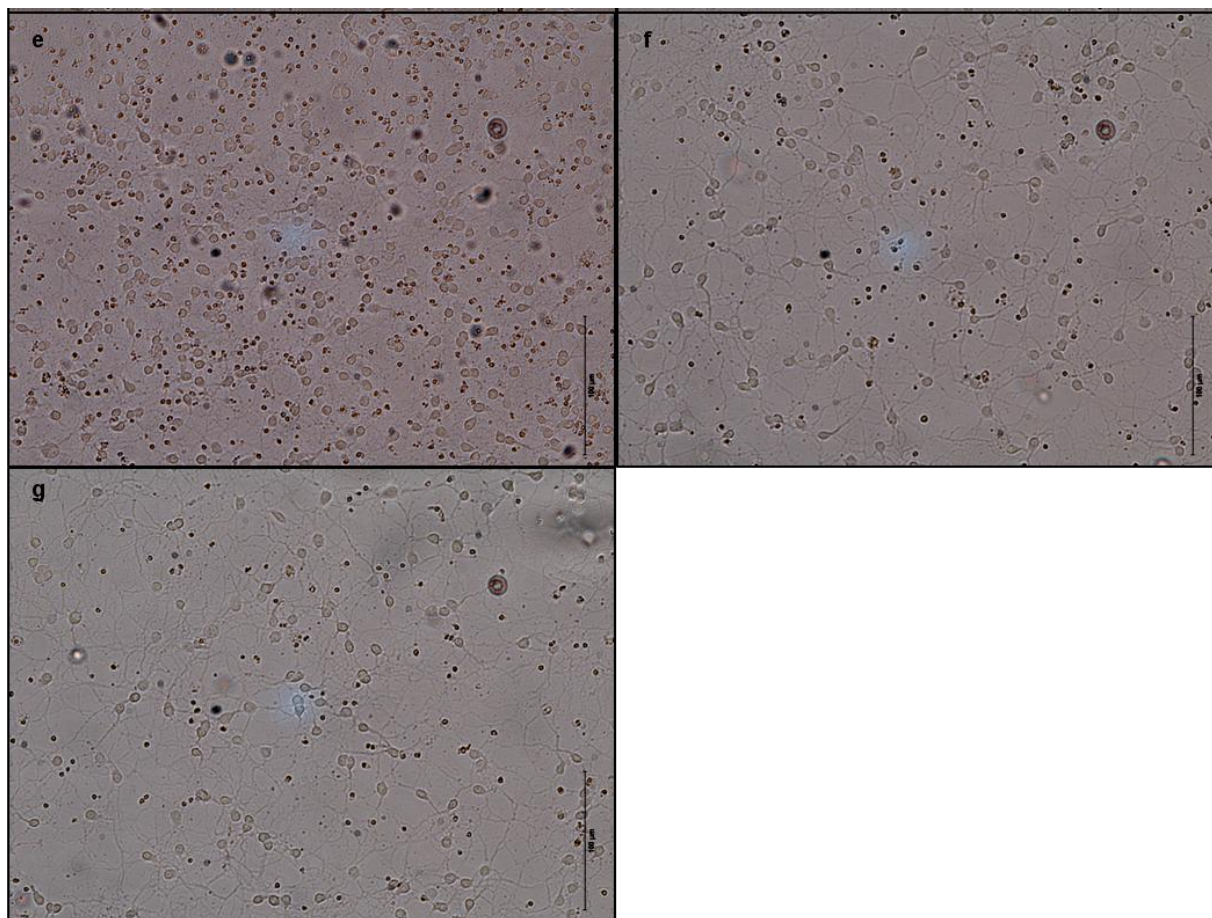


Figure 6 (continued from previous page) - Morphological aspects of cerebellar granule cells cultured for 11 days in medium with B-27 without antioxidants: (a) day1 in culture; (b) day 2 in culture; (c) day 3 in culture; (d) day 4 in culture; (e) day 5 in culture; (f) day 6 in culture; (g) day 7 in culture. Magnification: 40X.

Following the previous analysis, integrity and functionality of primary culture of cerebellar neurons were confirmed by immunostaining for tyrosine hydroxylase (TH) and β III-tubulin, two cytoplasmic proteins. TH takes part in the first step of the pathway that produces a group of hormones called catecholamines (namely dopamine), by which TH is considered as a marker of dopaminergic neurons^{28,72}. β III-tubulin on the other hand is a cytoskeleton tubulin protein almost exclusively found in neurons, and has been extensively used as a marker to identify neuronal cells from other brain cells such as glia, which do not express it⁶⁶. According to the images obtained (fig. 7), under normal conditions, cultured cerebellar granule cells at day 7 *in vitro* extended long, highly branched dendrites and were heavily connected to each other. The positive staining for both markers indicates that the present cells were effectively neurons and specifically refer to dopaminergic ones.

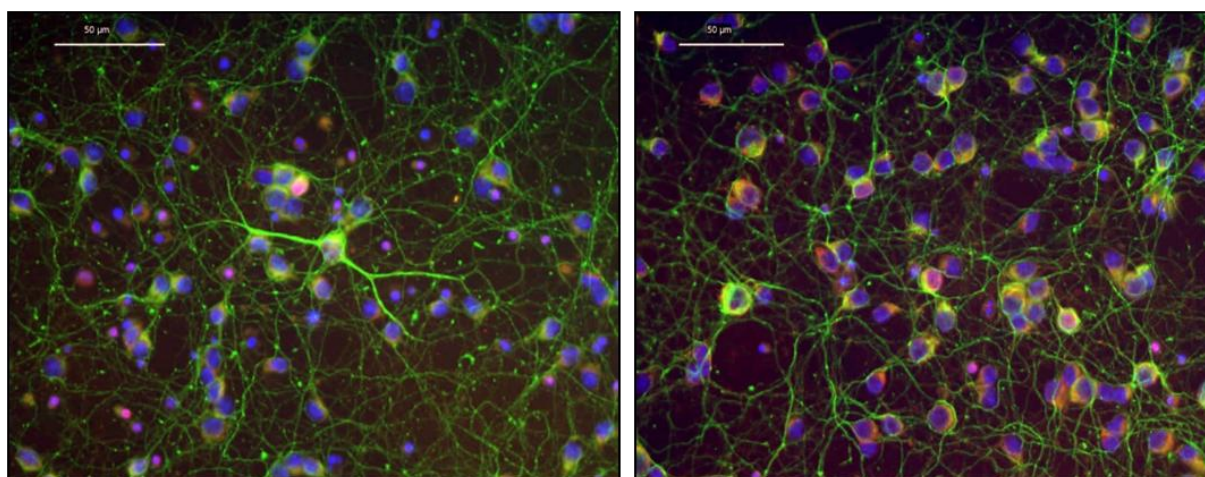


Figure 7 – Two different aspects of cerebellar granule cells stained with the neuronal specific marker β III-tubulin (green), the dopaminergic neurons marker tyrosine hydroxylase (red) and the nuclear marker DAPI (blue) after 7 days in culture. Magnification: 40X.

4.2 ESTABLISHMENT OF NEURODEGENERATION MODEL USING PRIMARY CULTURE OF NEURONS

In order to mimic a neurodegeneration condition, namely through an excitatory insult, glutamate was used to induce cytotoxicity, although H_2O_2 is commonly described as the toxic insult often used in cell models^{3,48,54}. Because the interaction between H_2O_2 and (poly)phenols exists, and because the culture media can never be completely removed, the use of glutamate instead of H_2O_2 eliminates or at least decreases that interference.

Throughout the literature, there is evidence that the sensitivity of cerebellar granule cells to glutamate depends on the number of days in culture⁷³, as they prevailed insensitive to all of the excitotoxic amino acids at days 3 and 5 in culture, and only became sensitive to toxicity induced by L-glutamine at day 8 in culture⁷⁴. This information is in accordance to the morphological mature state found on cerebellar granule cells at day 7 in culture. To establish the proposed neurodegeneration cell model with primary neurons, different concentrations of glutamate were tested in a range of 10 to 100 μ M, which is within the ranges referred to be found in plasma (30-100 μ M) and synaptic cleft (2-1000 μ M)²⁷. For this particular work, the selection of the most appropriate glutamate concentration was not only the concentration that caused considerable death in respect to the control condition, but also that enabled cells to have a significant response in the presence of the blackberries digested extracts.

The results obtained here revealed that the lowest concentrations of glutamate (10-50 μ M) present in the media for 24 h, did not alter cell viability, despite it has been already described that cerebellar granule cells viability was significantly decreased after exposure to 30 μ M, 100 μ M, and 3 mM of glutamate compared with the control cells not exposed⁷³. For the highest concentrations tested in this study (90 and 100 μ M), viability decreased for approximately 50% and 20%, respectively, in relation to the control (fig. 8). This significant

decrease made both of these concentrations a possible choice for the establishment of the neurodegeneration model.

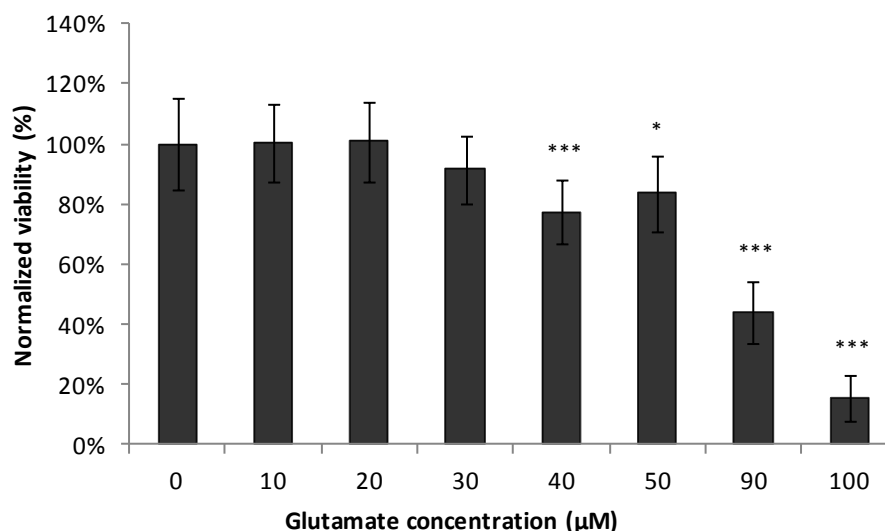


Figure 8 - Cell viability after 24 h incubation with different glutamate concentrations in order to optimize the most suitable concentration to establish the neurodegeneration model. Values are average \pm SD for at least three independent experiences. Statistical differences between treatments in relation to control are expressed as: * $p < 0.05$ and *** $p < 0.001$.

Nevertheless, accordingly to the similar model established for the neuroblastoma cell line developed in DSB Lab, to which the toxic stimulus (H_2O_2) is added at a concentration that allows an approximate reduction of 50% in cell viability³, 90 μM of glutamate was chosen. Morphological alterations were also observable when comparing primary neurons in culture with and without glutamate exposure. In the absence of glutamate, cerebellar granule cells grew normally in culture, properly extended their axons and formed dendrites and synaptic connections with each other (highlighted in fig. 9a). When, on the other hand, 90 μM of glutamate was present, its deleterious effects on neuronal morphology were recognized essentially in dendrites showing an irregular punctuate staining, which is indicative of neuronal damage (highlighted in fig. 9b). Moreover, there is an increase in the number of small rounded and dark cells, which represent dead cells (fig. 9b). As a matter of fact, immunostaining of hippocampal neurons evidences that the length and branching of dendrites in neural networks are severely reduced by the presence of glutamate⁷⁵. Regarding the cerebellar granule cells that contacted with neurotoxic concentrations of glutamate, it was previously demonstrated that they undergo two different death processes: a subpopulation collapse to acute necrosis during and immediately after the exposure, while the remaining neurons die from delayed apoptosis⁷³.

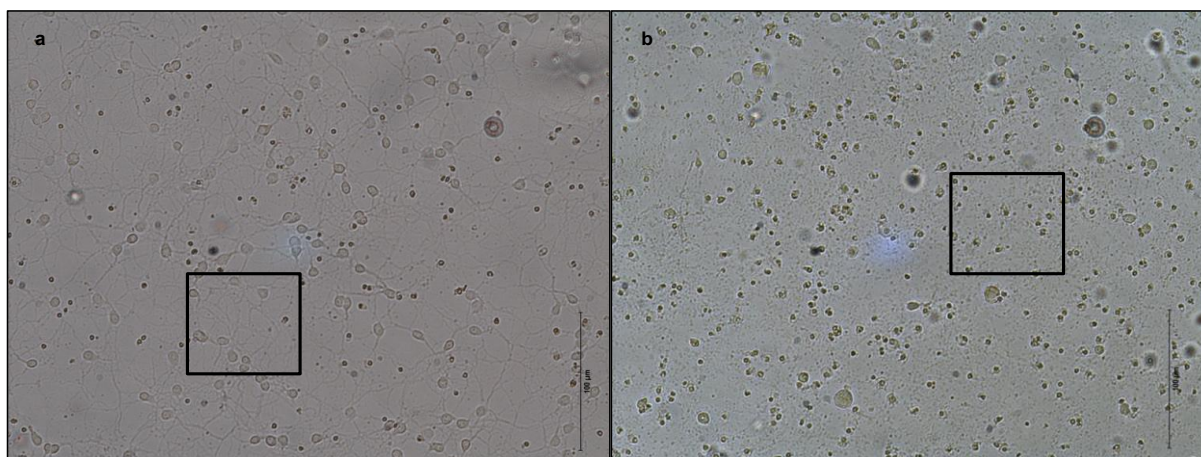


Figure 9 – Morphological differences between cerebellar granule cells at day 9 in culture (a) under control conditions and (b) in the presence of 90 μM of glutamate.

4.3 EVALUATION OF NEUROPROTECTIVE EFFECT OF BLACKBERRIES DIGESTED METABOLITES

Once established the neurodegeneration cell model with cerebellar granule cells, different concentrations of blackberries digested metabolites were tested in a range of 0 to 1 μg GAE mL^{-1} , in order to determine any cytotoxicity effects on the cells. This range corresponds to 0 to 6 μM GAE and is close to the physiological concentrations of certain phenolic components detected in human plasma, which is about 0 to 4 μM ^{3,45}. Applying this range of concentrations to the present neurodegeneration cell model, the biological significance will be increased in case of a neuroprotective effect. According to the results obtained, none of the concentrations tested revealed to be cytotoxic for primary neurons in culture, as viability was not significantly different from control conditions, meaning that all of them would be an adequate choice to be used in the neuroprotection study itself (fig. 10). However, because this work is in line with the neuroprotection studies that took place at DSB Lab within the neurodegeneration cell line model present at the time³, and in order to maximize a protective effect, we decided for the highest blackberries digested metabolites non toxic concentrations, which were 0.25, 0.5 and 1 μg GAE mL^{-1} , respectively.

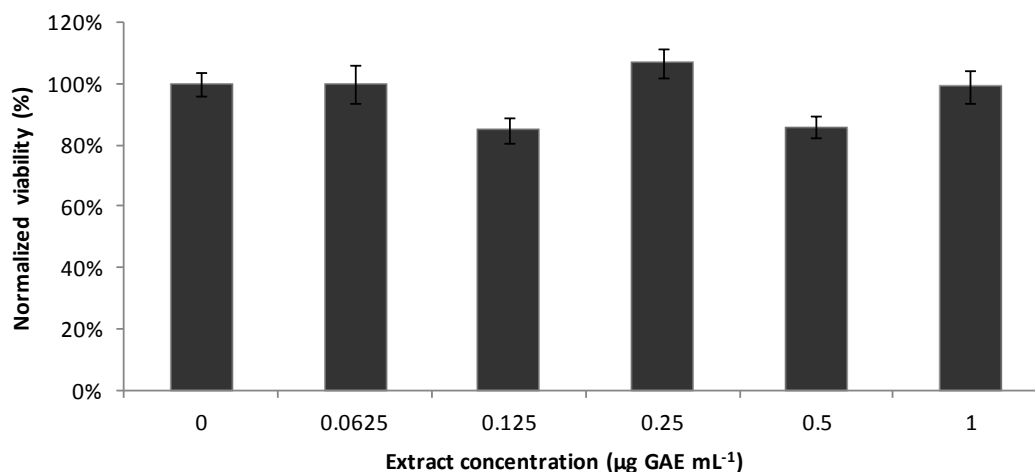


Figure 10 - Cell viability after 24 h incubation with different extract concentrations. Values are average \pm SD for at least three independent experiences.

These concentrations were then tested on the previously established neurodegeneration primary cell model by a pre-incubation for 24 h in the presence or absence of these digested metabolites, followed by a challenge with 90 μM glutamate for another 24 h (fig. 11). In samples treated with 90 μM of glutamate alone, only approximately 39% of neurons survived when compared to cells not challenged with glutamate. However, when digested extracts were present for 24 h preceding the glutamate insult, the viability rate increased considerably. The lowest concentration of blackberries digested metabolites tested (0.25 $\mu\text{g GAE mL}^{-1}$) was able to exert a similar neuroprotective effect to the other two higher concentrations (0.5 and 1 $\mu\text{g GAE mL}^{-1}$). They all significantly increased cell viability from 40% with glutamate alone to 61-67% with pre-incubation with digested extracts (up to 61-67%).

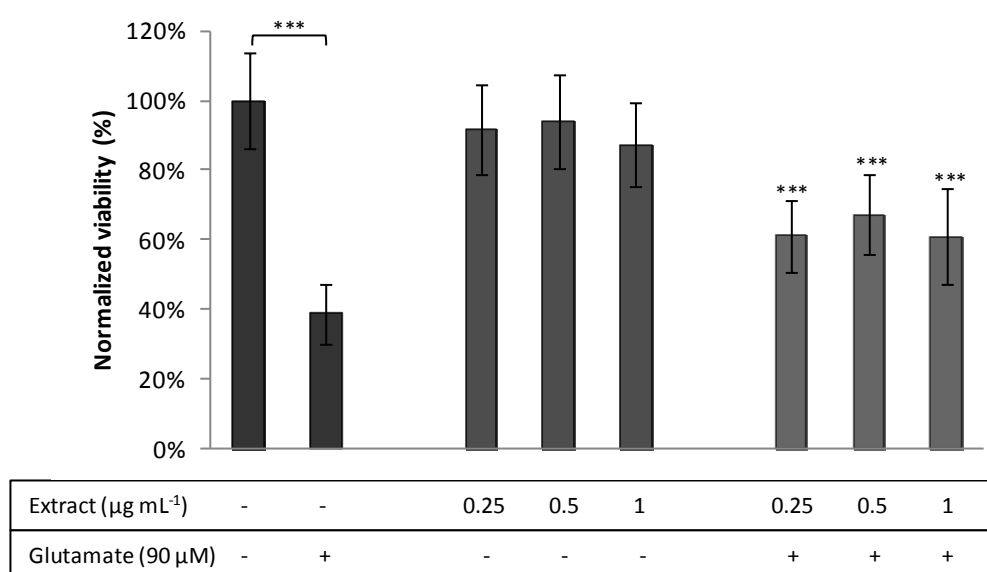


Figure 11 - Cytoprotective effect determination of blackberry digested extracts after 24 h incubation with extracts (0.25, 0.5 and 1 $\mu\text{g GAE mL}^{-1}$) followed by another 24 h incubation with glutamate (90 μM). Values are average \pm SD for at least three independent experiences. Statistical differences between treatments in relation to control are expressed as: *** $p < 0.001$.

Similar neuroprotective effects have been reported for cerebellar granule cells exposed to different types of extracts. For example, treatment of these cells with *Cymbopogon citratus* essential oil and *Ferula assa-foetida* extracts before and during exposure to glutamate, enhances the viability of cerebellar granule neurons in contrast with cytotoxicity and cell death induced by glutamate alone⁷³. On a similar study with coffee extracts, which has been recognized as a rich source of dietary compounds with potential to improve health, pre-incubation with brewed coffee extracts increased primary neurons survival under oxidative conditions⁷⁶.

4.4 ASSESSMENT OF MITOCHONDRIA BIOGENESIS

To gain some insight into the molecular mechanisms behind the neuroprotection previously demonstrated from blackberries digested metabolites, we investigated the levels of mitochondrial complexes expression through western blot analysis. It has been previously described that mitochondrial biogenesis is implicated on neuroprotective mechanisms⁷⁷.

According to the preliminary results obtained (fig. 12), and comparing lanes 1 and 2, some of the bands had decreased their intensity, suggesting that the respective complexes, namely I, II and IV might be affected by cellular stress conditions, such as excitotoxicity induced by glutamate. These complexes are also slightly affected with the incubation of digested extracts under concentrations of 0.25, 0.5 and 1 $\mu\text{g GAE mL}^{-1}$, represented on lanes 3, 4 and 5, respectively. However, when digested extracts were present for 24 h preceding the glutamate insult (lanes 6, 7 and 8) the band pattern seemed to be similar to the pattern present at the presence of the extracts alone, suggesting an improvement compared to glutamate alone (lane 2). Considering that the different levels of bands' intensity represent different levels of protein expression, it is suggested that under a neurodegenerative condition, complexes I, II and IV have a reduction on their expression, which is partially recovered when blackberries digested extracts are present.

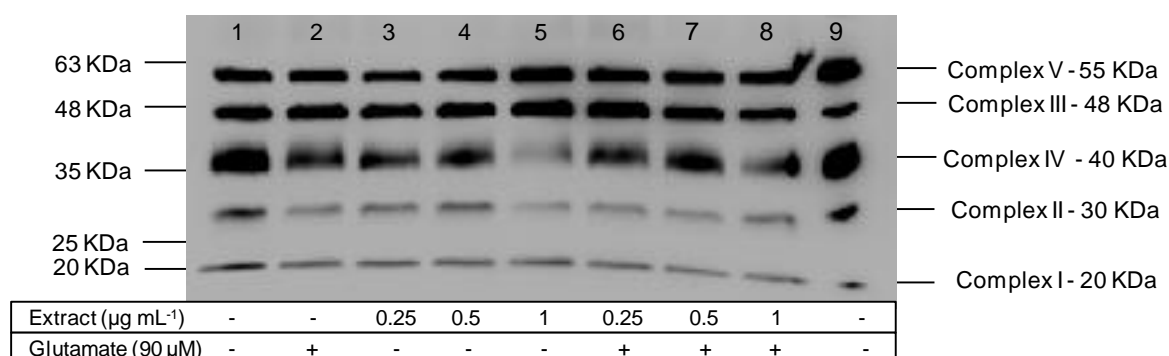


Figure 12 - Mitochondrial complexes profile of cerebellar granule cells after 24 h incubation with extracts (0, 0.25, 0.5 and 1 $\mu\text{g GAE mL}^{-1}$) in the absence of glutamate, depicted on lanes 1, 3, 4 and 5 respectively; or in the presence of 90 μM of glutamate for 24 h, depicted on lanes 2, 6, 7 and 8 respectively. Rat heart mitochondria was used as positive control (lane 9).

Mitochondrial damage is mainly associated with an increase in the generation of mitochondrial ROS (mtROS), to which lipids, proteins, OXPHOS enzymes and mtDNA are particularly vulnerable. As a consequence, damaged mtDNA causes a decrease in transcription and synthesis of the polypeptides associated with the ETC⁷⁸. Moreover, It has been shown that fragmentation of mitochondria in neurodegenerative diseases occurs from glutamate excitotoxicity, which is why it has been linked to mitochondrial dysfunction in both acute and chronic neurodegenerative disorders⁷⁹. Under those conditions of glutamate treatment, it has been observed that fragmented mitochondria appears as small round organelles in the damaged cells in contrast to long tubular mitochondria observed under control conditions²⁹.

Although the evaluation of protein expression for each of the complexes is important, a complete mitochondrial analysis should include an evaluation of the same complexes activities. In accordance with each specific neurodegenerative disease, the ETC complexes are differently affected. For instance, reduced activities of complexes I, III, and IV have been reported in platelets and lymphocytes from patients with AD and in post-mortem brain tissue⁸⁰. In case of post-mortem brain tissues from HD patients, decreases in enzyme activities of complexes I, II, III, and IV have been found^{81,82}. In patients and animal models of the HD disease, mitochondria were also described as excessively fragmented with decreased motility and respiration⁸³. Furthermore, complex I defects have been found in PD patients, and its activity was found to be reduced by 30% in brain and muscle tissues and in platelets from idiopathic PD patients⁸¹. Given the significant contribution of the mitochondria to cellular ATP synthesis, the high energetic demand of most cells requires these organelles to be highly dynamic, being biogenesis a particularly important process. Mitochondria biogenesis is regulated by a large number of co-activators and transcription factors. Although the proliferator-activated receptor coactivator-1 α (PGC-1 α) plays a major role as a master regulator of such process, its transcriptional activity seems to be primarily controlled by SIRT1, the silent mating type information regulation 2 homolog 1, whose activation has been demonstrated to be mediated by several (poly)phenols. Therefore, some of them are currently being investigated as potential inducers of mitochondrial biogenesis, such as resveratrol, quercetin and hydroxytyrosol⁸⁴. In addition to those, other (poly)phenols effects on mitochondrial complexes expression have been tested. For example, quercetin supplementation reversed inhibition of respiratory chain complexes II, IV and V induced by 3-nitropropionic in an induced cell model of HD⁷⁸. Caffeic acid treatment on the other hand restored the levels of ETC complexes I, II and IV in mice with fatigue syndrome⁷⁸. Resveratrol significantly restored the activity of mitochondrial respiratory complexes and decreased CYT C (cytochrome c) release, in a rat middle cerebral artery occlusion model of brain ischemia⁷⁸, and also significantly increased the T regulatory cells production and stimulated its mitochondrial biogenesis in high fat diet mice⁷⁷. Moreover, epicatechin-rich cocoa increased expression of complexes I and V protein levels in patients with type 2 diabetes and heart failure and curcumin increased expression of mitochondrial respiratory complex IV in the brain of senescence-accelerated mouse-prone 8⁸⁴.

As mentioned before, the results obtained for the mitochondrial analysis represent only a preliminary attempt, which suggests the involvement of complexes I, II and IV in glutamate-induced neurodegeneration processes. Therefore, future immunoblot replicates for OXPHOS should be performed, so that more precise and consistent results can be obtained regarding the involvement of glutamate and blackberries extracts on mitochondria. Also genes regulating mitochondrial biogenesis should be evaluated.

4.5 PCR

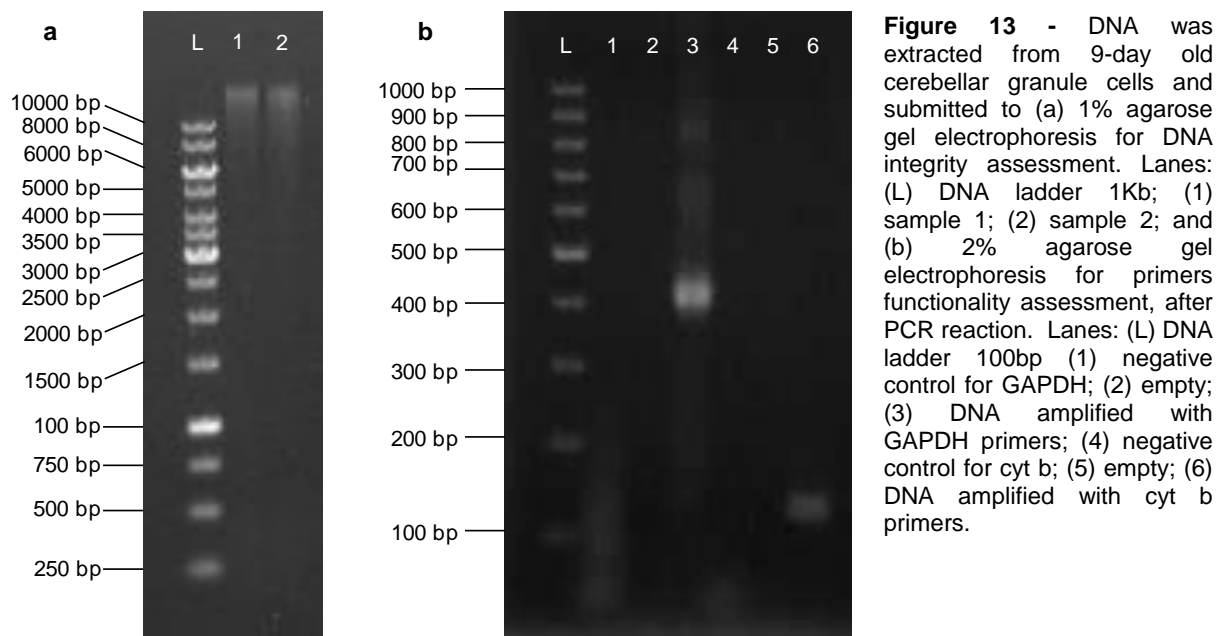
As mentioned before, after protein analysis through western blot, the analysis at DNA level was the next phase, in which preliminary tests on DNA were performed, so that integrity and amplification by the selected primers was assessed. Analyzing the results regarding DNA quantification (table 1), it is evident that the extraction yield was not very high, although the amount of DNA obtained was enough for the purpose. In addition to quantification, two ratios were determined: the ratio between nucleic acids and proteins (through the absorbances measured at 260 and 280 nm) and the ratio between nucleic acids and sugars (through absorbances measured at 260 and 230 nm) (table 1). Taking into consideration that the ideal ratios should range from 1.8 to 2, it can be assumed that in terms of protein content in the sample, the 260/280 ratio is near the optimal values. On the contrary, the 260/230 ratio is far from the ideal, which is why we decided to test the possible interference of sugars on the DNA sample.

Table 1 - Two biological samples of DNA extracted from 9-day old cerebellar granule cells. Further quantification and ratios (nucleic acids/proteins (260/280) and nucleic acids/sugars (260/230) determination was performed.

	Sample		Average \pm SD
	1	2	
DNA concentration (ng μL⁻¹)	25	22.7	23.9 \pm 1.6
Ratio 260/280	1.63	1.71	1.670 \pm 0.057
Ratio 260/230	0.91	1.07	0.99 \pm 0.11

After a previous assessment on DNA quantity and its ratios, the next parameter included a simple analysis at the integrity of the two DNA samples previously extracted, so that sugars interference could be analyzed. According to the 1% agarose gel obtained, DNA samples were considered acceptable to be used for the amplification process, as each sample was mostly represented by a defined single band (with more than 10000 bp) on each lane (with some smear), meaning that DNA is integrate enough to be considered for the purpose (fig. 13a). In the gel obtained to assess primers functionality, as well as the usefulness of the amount of DNA extracted (fig. 13b), it is possible to observe that the lanes corresponding to the negative controls (lanes 1 and 4) do not show any amplification, meaning that there is no contamination. Nonetheless, there was amplification of the specific PCR products by each pair of primers (lanes 3 and 6). For GAPDH gene (fig. 13b, lane 3), the amplification was not completely specific, as some unspecific amplification was observable, suggesting the amplification of other higher molecular weight products. As a preliminary work, the results obtained mean that some adjustments and optimizations on the PCR reaction conditions (primers and MgCl₂ concentrations and annealing temperature) still needed to be performed.

For cyt b (fig. 13b, lane 6), it is possible to observe a tenuous solid band, suggesting that the amplification was specific and that the amount of amplified product was not very high. Thus, it seems that the amplification conditions, in terms of annealing temperature and primer design were adequate.



Although it was not possible to proceed with the quantitative PCR originally defined (due to the absence of mice provision and lack of time), but still on course, further work can be predicted. Besides new DNA extraction for each condition tested (incubation with the three different concentrations of blackberries digested extracts under a glutamate stimulus), it is expected to apply the specific forward and reverse primers (same as already used for conventional PCR) for both mitochondrial (cyt b) and nuclear (GAPDH) genes. Amplification of DNA with primers for different cell location genes, will allow to compare the number of copies present from each other, and by that, to understand if there is some differential number of copies of mtDNA, suggesting a different number of mitochondria in cells submitted to the different treatments. In that sense, two overviews are possible. Comparing two experimental conditions, if mitochondrial DNA is being more amplified, mitochondria should be suffering biogenesis, as it is expected to happen when cells are incubated with blackberries digested extracts followed by glutamate exposure, in comparison with cells only treated with glutamate. On the other hand, if mitochondrial DNA is being less amplified, it would probably mean that mitochondria are reducing their population, what is expected when cells are treated with glutamate, in comparison with control cells. The PCR results will hopefully come in line with the ones obtained at protein level (with western blot analysis), and will enable to globally understand the mitochondrial biogenesis.

5. FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

Health and food has always been strictly related, although only recently its interaction has been properly recognized. In that context, fruits and in particular berries have been associated to a good outcome of different diseases, from neurodegenerative, to cardiovascular and cancer^{13,15,49}. Therefore, with this work, primary cultures of neurons have been established for the first time in DSB Lab, which were used to develop a neurodegeneration model where blackberries digested metabolites were tested on. And as a major result, we observed that the extracts (0.25, 0.5 and 1 µg GAE mL⁻¹) increased cell viability under a stress condition (generated by 90 µM of glutamate), validating the previous results obtained for the same extracts in a neuronal cell line at DSBLab⁴⁸. Moreover, the potential of digested extracts to ameliorate mitochondrial complex IV expression after treatment with glutamate was suggested.

When it comes to further work, some future perspectives naturally arise:

- It would be interesting to further analyze primary neurons in terms of their morphology, specifically in respect to neurite's size and thickness. Specific virtual measuring tools would be used to try to correlate the data obtained with neurodegeneration processes, as it is described that for neurological conditions in which neurons health is compromised, its' neurites reflect the damage by diminishing their length and thickness⁷⁶;
- In order to complement the qPCR analysis, a determination of the expression levels of the main proteins involved in mitochondrial fusion and fission processes by western blot could be performed. By doing that, a clearer association between a mitochondrial process and specific cellular conditions could be established. Those proteins would be Drp1, which mediates fission, and OPA1, Mfn1 and Mfn2, which mediate fusion. In addition to that, enzymatic activity of each complex could also be determined to complement the complexes analysis;
- Because the neurological system is far more complex and because we always try to mimic this system *in vitro* as closer to the reality as possible, it would probably be an interesting upgrade to work on co-cultures of primary neurons and microglia/astrocytes, as microglial cells, the major resident immunoreactive population in the brain, are actively involved in the pathological inflammatory events. This chronic inflammation in the brain plays in turn an important role in the pathogenesis of neurodegenerative disorders⁷²;
- This work also open new avenues for the host lab, since it was implemented a neurodegeneration cell model based on primary neurons, a more physiological model than cell lines and therefore it becomes possible to test and validate effects for other bioactive compounds. In particular, it was already tested for study the effects of human bioavailable polyphenols metabolites⁸⁵ for their potential neuroprotective capacity.

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